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Identification and characterization of the *doublesex* gene of *Nasonia*

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Abstract

The *doublesex* (*dsx*) gene of the parasitic wasp *Nasonia vitripennis* is described and characterized. Differential splicing of *dsx* transcripts has been shown to induce somatic sexual differentiation in Diptera and Lepidoptera, but not yet in other insect orders. Two spliceforms of *Nasonia dsx* mRNA are differentially expressed in males and females. In addition, in a gynandromorphic line that produces haploids (normally males) with full female phenotypes, these individuals show the female spliceform, providing the first demonstration of a direct association of *dsx* with somatic sex differentiation in Hymenoptera. Finally, the DNA binding (DM) domain of *Nasonia* dsx clusters phylogenetically with dsx from other insects, and *Nasonia dsx* shows microsynteny with *dsx* of *Apis*, further supporting identification of the *dsx* orthologue in *Nasonia*.

Keywords: DM domain genes, *doublesex*, haplodiploidy, Hymenoptera, sex determination.

Introduction

The role of the transcription factor doublesex (dsx) in sex determination was first described in *Drosophila melanogaster* (Baker & Wolfner, 1988). Differential splicing of male and female *dsx* mRNA results in sex-specific dsx proteins, which regulate downstream somatic sexual dimorphism (Baker & Wolfner, 1988). The dsx protein is characterized by two functional domains, an N-terminal DNA binding domain (DM domain or OD1) and a C-terminal dimerization domain (dsx dimer or OD2). The DM domain is a zinc module, with conserved amino acid residues for zinc chelating and promoter binding (Zhu *et al*., 2000). The dsx dimer is an alpha-helical motif which, by promoting dimerization of dsx, enhances DNA recognition by the DM domain (Bayrer *et al*., 2005).

Strong conservation of the DM domain occurs between *D. melanogaster* dsx (DMdsx) and the sex determining protein mab-3 in the nematode *Caenorhabditis elegans*. Function of the gene in these divergent organisms is sufficiently conserved that DMdsx can partially rescue male-specific development in *mab-3* deficient worms (Raymond *et al*., 1998). Furthermore, the mammalian *Dmrt-1* gene transcribes a DM domain protein that is differentially expressed in testes, and mutations in this gene suggest that it plays an important role in male sexual differentiation (Raymond *et al*., 2000). Recently, *dsx* orthologues have been detected in a variety of insects. *Doublesex* orthologues have most extensively been described in Diptera, including *Anastrepha obliqua* (Ruiz *et al*., 2005), *Anopheles gambiae* (Scali *et al*., 2005), *Bactrocera tryoni* (Sherman & Frommer, 1998) and *Musca domestica* (Hediger *et al*., 2004), but also have been found in Lepidoptera (*Bombyx mori*, Ohbayashi *et al*., 2001) and Hymenoptera (*Apis mellifera*; Cho *et al*., 2007), as all have a characteristic strongly conserved DM domain. However, in only a few cases have spliceform differences between males and females been shown, eg in *Ap. mellifera* (Cho *et al*., 2007) and functional assays are restricted to *Drosophila* (eg Baker & Wolfner, 1988; Raymond *et al*., 1998) and to a lesser extent to Lepidoptera (Suzuki *et al*., 2005). A current hypothesis predicts higher conservation at the base of sex-determining gene cascades, ie *dsx*, but divergence at the level of upstream primary signals (Wilkins, 1995).

Hymenoptera, including the honeybee *Ap. mellifera* and the jewel wasp *Nasonia*, have haplodiploid sex determination. Males are haploid and develop parthenogenetically from

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unfertilized eggs, whereas females are diploid and develop from fertilized eggs. For over 60 years it has been known that different sex-determining mechanisms exist within the Hymenoptera (Whiting, 1943). In haplodiploids, a relatively common mechanism is complementary sex determination (CSD). Gender is genetically determined by a single locus with multiple alleles: individuals that are heterozygous at this locus develop into females, whereas hemizygotes and homozygotes develop into haploid and diploid males, respectively (Whiting, 1943; Crozier, 1971). This mode of sex determination has now been shown for more than 60 species (van Wilgenburg *et al*., 2006) and for the honeybee the *complementary sex determiner* (*csd*) gene was identified and cloned (Beye *et al*., 2003). However, it is clear that sex determination in some groups, such as the large parasitoid wasp group Chalcidoidea to which *Nasonia* belongs, cannot be explained by CSD, because upon inbreeding homozygous diploids still develop into females (Skinner & Werren, 1980).

The parasitic wasp *Nasonia* has been extensively studied genetically and is rapidly being recognized as a model system in evolutionary and developmental biology (Werren & Stouthamer, 2002; Shuker *et al*., 2003; Pultz & Leaf, 2003; Beukeboom & Desplan, 2003; Werren *et al*., 2004; Ferree *et al*., 2006; Lynch *et al*., 2006). It has been known for a long time that its sex determination is not governed by CSD (Whiting, 1967; Skinner & Werren, 1980), but until recently little progress had been made in elucidating its mode of sex determination (Beukeboom, 1995). Recently, several studies have reported on the genetics of sex determination in *Nasonia* (Beukeboom & Kamping, 2006; Trent *et al*., 2006), including the characterization of genetic strains that produce gynandromorphic phenotypes (Beukeboom *et al*., 2007a; Kamping *et al*., 2007).

The recent advent of the *Nasonia* genome sequence (Werren *et al*., 2004) now provides avenues for identification of genes involved in sex determination, and for investigation of their potential role in *Nasonia* sex-determination pathways and the evolution of genetic sex-determining mechanisms in general. Here we report the identification and characterization of a *dsx* orthologue in *Nasonia*. We show that *Nasonia* dsx clusters phylogenetically with dsx in other insects, is differentially spliced in males and females and is associated with sex determination in haploid individuals from a gynandromorph-producing strain of *Nasonia*. We have also investigated the presence and sequence divergence of *dsx* in related species of *Nasonia*.

Results

Computational detection of the Nasonia dsx *orthologue*

A gene containing a DM domain-coding sequence that showed striking homology to *dsx* of other insects was originally detected during efforts to clone genes involved in morphological sex-specific differences amongst *Nasonia* species (J. H. Werren *et al*., unpubl. data). The putative coding region of this gene, provisionally termed DM1, was represented in a fully sequenced bacterial artificial chromosome of *Nasonia girault*i (NG, GenBank accession number AC185330). Subsequently, the *N. vitripennis* (NV) genome was bioinformatically screened with the DM domain-coding sequence and, in addition to finding the NV DM1, three additional DM-domain genes were detected, termed DM2, DM3 and DM4. These four DM domain-containing genes were found in the following scaffolds: DM1, scaffold 23; DM2, scaffold 53; DM3, scaffold 62; DM4, scaffold 6 (NV genome version 1.0; HGSC at Baylor College of Medicine). Besides giving the strongest BLAST match to any insect *dsx* genes, the DM1 gene also contains a second conserved coding sequence characteristic of dsx proteins: the dsx dimerization domain (dsx dimer). Only one gene containing the dsx dimer was found in each of the five insect genomes searched (see below). Further analysis of scaffold 23, involving the immediate flanking sequences of DM1 revealed that the DM1 is flanked upstream by a *prospero* orthologue and downstream by an *elongase* gene (data not shown). Microsynteny of *prospero* – *dsx* gene order is also present in the genomes of *Ap. mellifera*, *Tribolium castaneum* and *An. gambiae*, but not in *D. melanogaster*.

Four different DM-domain genes were also found in the genomes of *D. melanogaster* and *Ap. mellifera*, whereas only three were found in the genomes of *T. castaneum* and *An. gambiae*. Phylogenetic analysis using amino acid sequences of the DM domain showed four gene clusters, each containing a single gene from each species (Fig. 1). These phylogenetic clusters are consistent with the overall gene architecture, as indicated by other identifiable structural markers, eg the dsx dimer in the dsx cluster and the DMRTA motif (pfam 03474) in the dsx-mab 93B cluster (data not shown). The *Nasonia* dsx groups with dsx of the other species with strong support. The *Nasonia* dsx DM domain has a number of derived amino acid replacements that are conserved in other species, including the honeybee (Fig. 2). In addition to *dsx*, all five species share genes homologous to dsx-mab 93B and dsx-mab 99B genes (DM2 and DM4, respectively, for *Nasonia*), the latter being the less divergent gene, indicating stronger selective pressure to maintain the amino acid sequence. The fourth gene dsx-Mab 11E (DM3 for *Nasonia*) was not found in the genome of *T. castaneum* and *An. gambiae*. As most insect genomes are still in draft stage, at this point it is difficult to be sure whether dsx-mab 11E has not been sequenced or it was really lost during *T. castaneum* and *An. gambiae* evolution. However, it appears that four DM domain genes is the ancestral condition in holometabolous insects.

Gene architecture, genomic microsynteny and phylogenetic analysis of conserved regions of established *dsx* genes lead us to the conclusion that DM1 is the *Nasonia* orthologue of *dsx*.

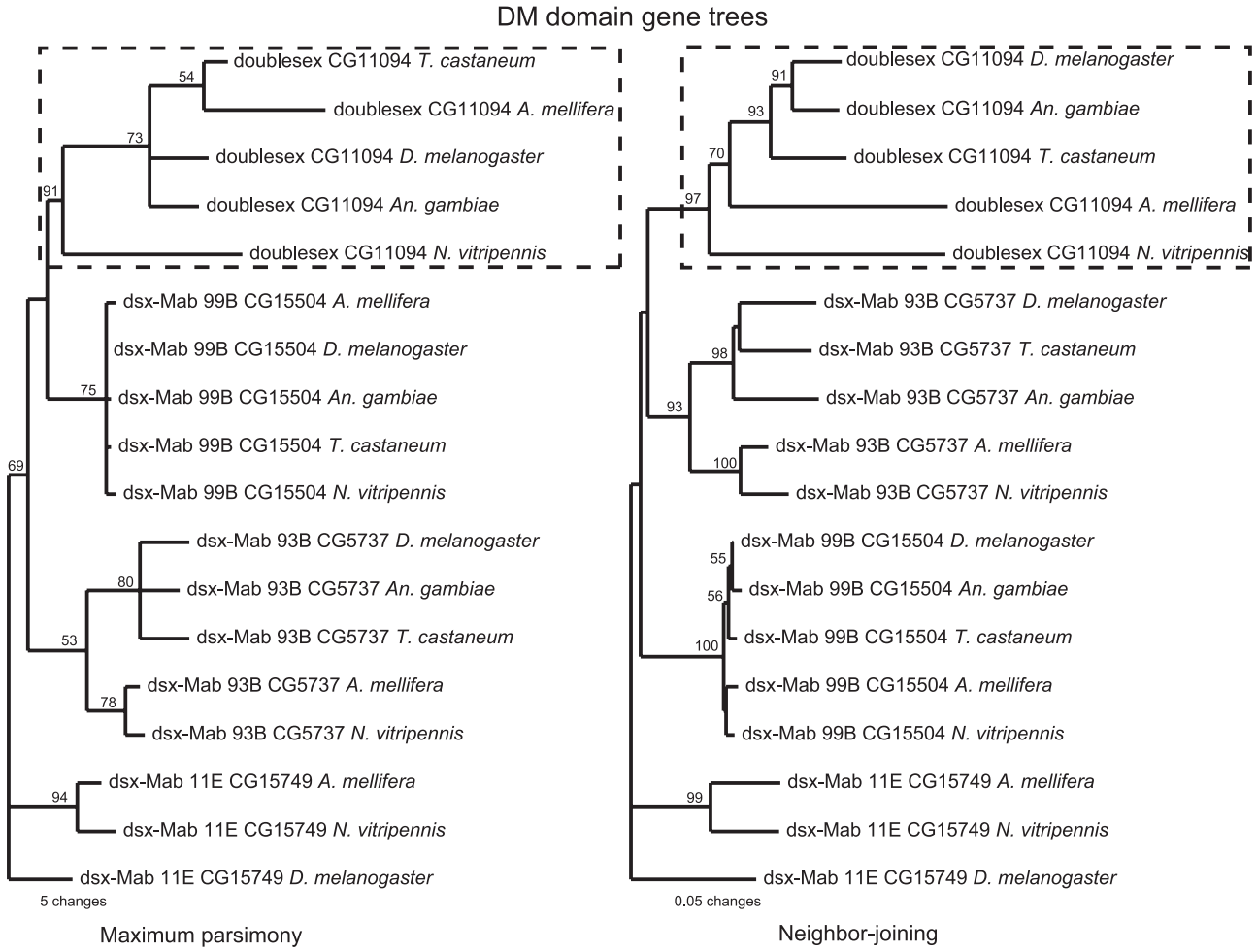

Figure 1. DNA binding (DM) domain gene tree. *Nasonia* doublesex (dsx) clusters with dsx of other insects. The most parsimonious tree (consensus of 20 trees with 222 steps) and a neighbor-joining tree obtained using the amino acid sequences of the DM domain region (67 total characters, 42 that are parsimony-informative) are shown with bootstrap supports above the branches. Many of the DM domain genes used in this analyses are predicted gene models.

Sex differential splicing of Nasonia dsx *mRNA*

Male and female forms of *dsx* were detected using primers designed for the predicted 3′ untranslated region (UTR) and the DM-domain exon. Results of these experiments show that from female mRNA an approximately 500 bp fragment is predominantly amplified, whereas in males the amplicon using the same primer set is approximately 110 bp longer (Fig. 3).

The male and female spliceforms were confirmed by sequencing of the rapid amplification of 3′ cDNA ends (3′-RACE) products generated for NV (AsymC and HiCD12 strains) and NG. Full-length transcripts were determined for the NV strain HiCD12 by sequencing of the 5′ RACE products. For the second NV strain, AsymC, and for NG, several reverse-transcription PCR (RT-PCR) reactions with different primer combinations confirmed all intron–exon boundaries including the most 5′ exon (Fig. 4).

The mature *Nasonia* male *dsx* mRNA is composed of five exons, with a single large exon 5M of 1082 bp (hereafter sizes are for NV). This last male exon includes the C-terminal coding sequence, starting from the dsx dimer region and does not undergo further splicing. The first four exons of *Nasonia* female mature *dsx* mRNA are identical to the male isoform. The *Nasonia* female *dsx* mRNA, however, is derived from six exons. Exons 5F and 6F are derived from the same exact genomic region as the last male exon (exon 5M) but are interrupted by an intron of 108 bp that is not spliced out in the male mRNA, resulting in two exons of 147 bp (exon 5F) and 827 bp (exon 6F), respectively. Sex-specific differences in dsx protein sequence are caused by female-specific splicing of the last exon resulting in a different stop codon (see below).

To summarize, in *Nasonia* females an intron of 108 bp is spliced out of the primary transcript, whereas in males no splicing of this intron occurs (Fig. 4). This process of generating the male-specific dsx isoform has not been described for other insects. In other insects, including *Ap. mellifera* (Cho *et al.*, 2007), *D. melanogaster* (Baker &

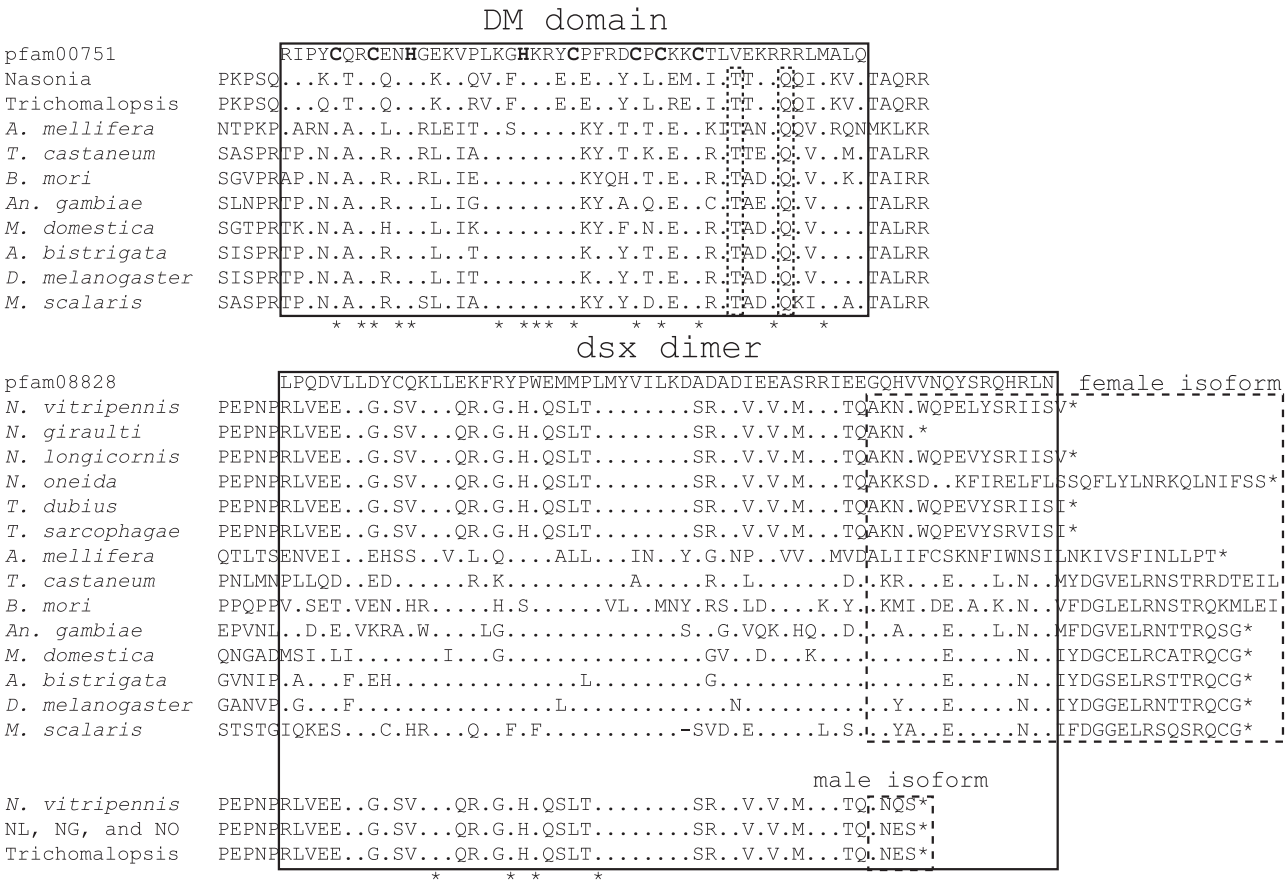

Figure 2. Amino acid sequence alignment of the *Nasonia* doublesex (dsx) and those of other insects. Only the two conserved regions are presented in the figure, including the DNA binding (DM) and the dsx dimer. The zinc chelating residues in the DM domain are in bold in the reference pfam sequence (Finn *et al.*, 2006). Putative conserved residues that distinguish the dsx DM domain from the DM domain of other proteins are shown in dotted boxes. The female isoforms were used for the dsx dimer region alignment. The predicted amino acid sequences of the 3′ region of the male isoform are shown for *Nasonia* and *Trichomalopsis*.

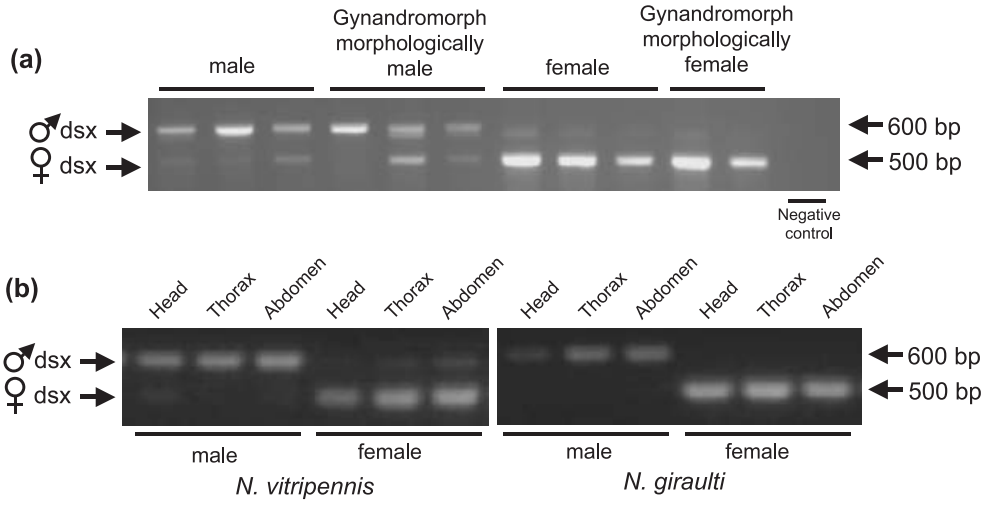

Figure 3. Male and female spliceforms of *Nasonia* doublesex (dsx). Reverse-transcription PCR of males, females and gynandromorph RNA. Arrows indicate male-specific and female-specific splice fragments. Lanes 1–3: adult male; lanes 4–6: adult HiCD12 haploid gynandromorph, morphologically male; lanes 7–9: adult female; lanes 10–11: adult HiCD12 haploid gynandromorph, morphologically female; lane 12: negative control; lane 13: 100 bp molecular marker.

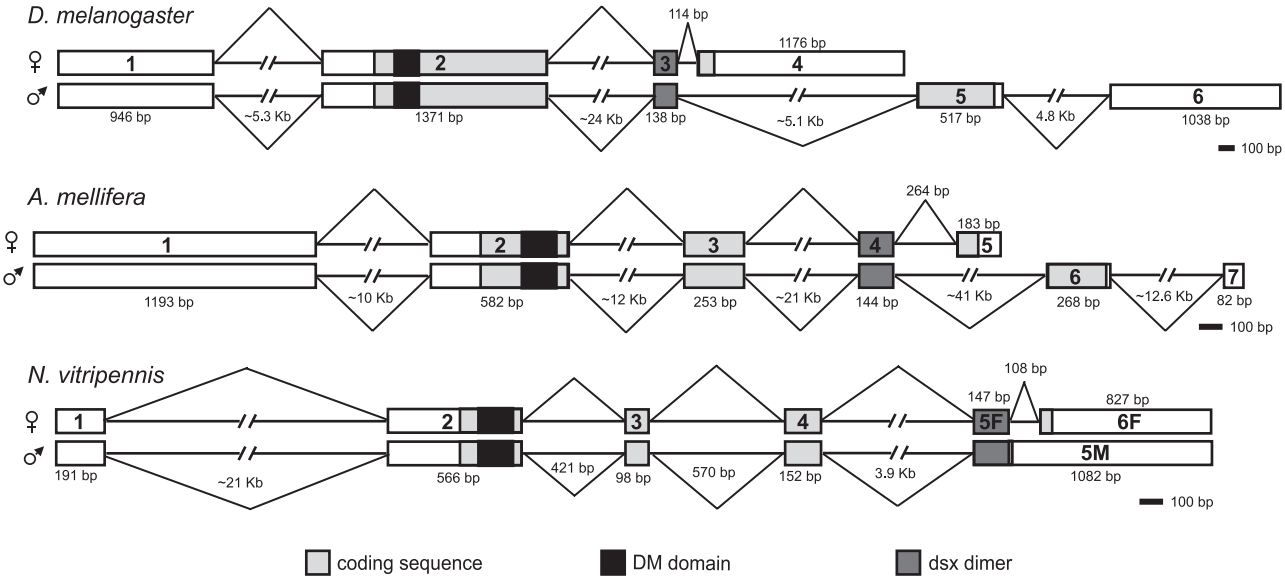

Figure 4. Insect *doublesex* (*dsx*) gene models. The *Nasonia* dsx male and female spliceforms are compared to *dsx* of *Drosophila melanogaster* and *Apis mellifera*. See text for details.

Wolfner, 1988) and *B. mori* (Ohbayashi *et al*., 2001), the male isoform is generated by an alternative splice that completely skips the female-specific exon (see Fig. 4 for a comparison).

RT-PCR conducted for different life stages (embryos, larvae, pupae and adults), and for different adult body parts (head, thorax and abdomen) showed that both sexes predominantly express their sex-specific form in all stages and body parts tested (Fig. 3B shows adults). The female form was often detected in independent experiments as a weaker band in males, whereas the male form was rarely seen in females. The presence of the female form of *dsx* in males was also noted in *Ap. mellifera* (Cho *et al*., 2007).

Interestingly, for NV in both males and females a 564 bases longer 3′ UTR transcript was also identified (but not for NG). Apparently a second poly(A) site can be used in both males and females to generate longer *dsx* mRNAs. As these longer forms are observed in spliced mRNA, ie they do not include intronic sequences, we believe that these products are not the result of genomic contamination, but represent a genuine *dsx* mRNA. However, comparison of these 3′ UTR sequences to the genomic sequence of NV shows that a stretch of 25 As is present in the NV genomic sequence. Therefore, the actual alternative poly(A) site could be even more downstream, but was not detected because of the annealing of the oligo-d(T) to the internal A-tract upon performing 3′-RACE. In addition, a smaller transcript that skips exon 2 (Fig. 4), ie a spliceform that joins directly exons 1 and 3, was consistently observed as a clear second band in RT-PCR in both sexes. This is puzzling because exon 2 codes for the DM domain and it also has the start codon (an alternative start codon maintains the frame). Obviously, these alternative transcripts will need further characterization and are not described in more detail here. It is worth noting that evidence for more than two splicing variants of *dsx* have been shown previously, eg for *D. melanogaster* (Baker & Wolfner, 1988) and *Ap. mellifera* (Cho *et al*., 2007).

dsx *mRNA splice variants in gynandromorphs*

Gynandromorphism (individuals with both male and female structures) has been previously described in *Nasonia*. A line (HiCD12) has been characterized that produces gynandromorphs from unfertilized (haploid) eggs which range from single female structures (eg antennal segment) in an otherwise male morphology to haploids with a complete female morphology (Beukeboom *et al*., 2007a; Kamping *et al*., 2007). To evaluate the involvement of *dsx* in hymenopteran sex determination, we analysed haploid individuals from the HiCD12 line that were either completely morphological male or female for the presence of male and female specific *dsx* splice variants. We found that morphological male gynandromorphs always express the male-specific splice variant at a considerable level, sometimes together with the female-specific variant, whereas haploid females express mostly or only the female transcript (Fig. 3A). Presence of the female-specific spliceform in morphological males of the gynandromorphic line may be because of the presence of female-like structures in these individuals that were not apparent from their outer morphology. As stated above, however, haploid males from the nongynandromorphic strain NV AsymC can also contain low levels of female-specific splice variants of *dsx* mRNA (see Fig. 3B). In contrast, females of either strain show a clear predominant female-specific spliceform, confirming the female specificity of this splice variant.

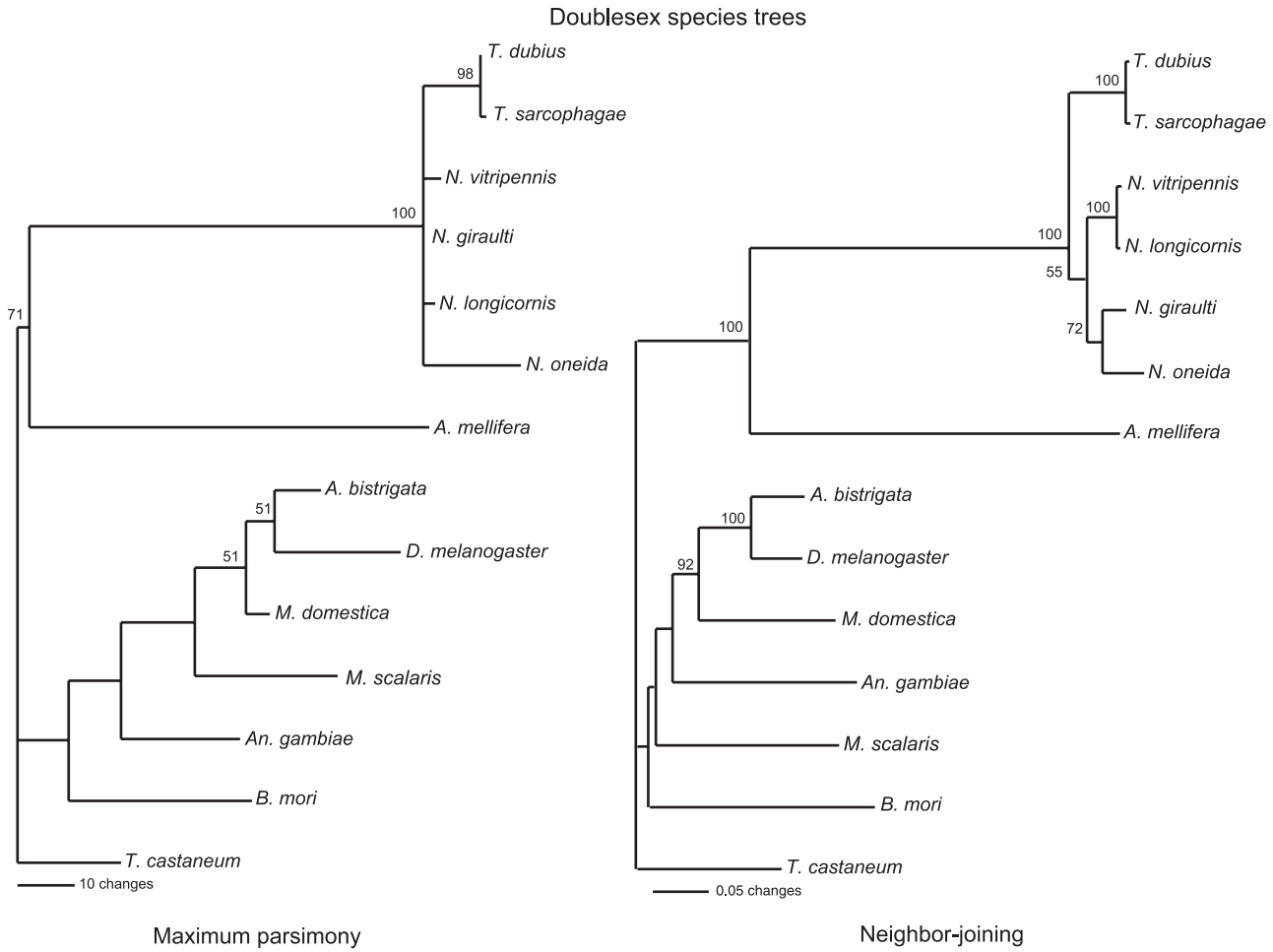

Figure 5. An insect species trees based on the conserved doublesex (dsx) amino acid sequences. The most parsimonious tree (consensus of 20 trees with 335 steps) and a neighbor-joining tree obtained using the combined amino acid sequences of the DNA binding (DM) domain region and the dsx dimer region (138 total characters, 89 that are parsimony-informative) are shown with bootstrap support above the branches.

The difference between a normal female and a phenotypic female from the gynandromorph line is that the former are diploid, whereas the latter are derived from haploid unfertilized eggs, yet develop somatically into females. These data clearly support an association for *dsx* in *Nasonia* sex-specific phenotypic differentiation, independent of ploidy.

Predicted dsx proteins in Nasonia *and other insects*

In *Drosophila* and other organisms, dsx has two characteristic domains: a DNA binding domain (DM or OD1) and an oligomerization domain (dsx dimer or OD2). We have sequenced the genomic region of all coding exons of *dsx* of two additional *Nasonia* species as well as of two *Trichomalopsis* species, a closely related pteromalid wasp. The two conserved regions of the predicted dsx proteins were aligned with known dsx sequences from eight other species: the hymenopteran *Ap. mellifera*, the dipterans, *Anastrepha brisgata*, *D. melanogaster*, *An. gambiae*, *M. domestica*, *Megaselia scalaris*, the lepidopteran *B. mori* and the coleopteran *T. castaneum* (Fig. 2). Parsimony and neighbor-joining dendrograms of this alignment shows that the combined protein sequence groups *Nasonia* dsx with *Ap. mellifera* dsx (Fig. 5), as expected based on phylogenetic relationships. *Apis* and *Nasonia* did not cluster together when only the DM domain was used (Fig. 1), reflecting the amino acid sequence divergence of this domain in *Nasonia*. The Hymenoptera combined dsx domains show longer branch lengths than found in other insects (Fig. 5), probably caused by an elevated divergence rate.

Examination of the DM domain consensus (pfam00751, Fig. 2) reveals amino acids that are conserved across the DM domains shown, relative to the consensus for DM domains. Most of these conserved amino acid residues are not dsx specific, but are common to other DM domain-containing proteins and are important for zinc binding (Zhu *et al.*, 2000). However, examination of the DM-domain sequences also

reveals that two highly conserved amino acids are shared among all insect dsx. An insect dsx shared threonine (T) occurs four amino acids upstream of a conserved glutamine (Q) in the second half of the DM domain (Fig. 2). A third highly conserved glutamic acid (E) occurs five amino acids upstream of the conserved T. Only *Trichomalopsis* shows a variant at this position, with arginine (R) in place of E. Both are polar amino acids with strong hydropathy indices, whereas glutamic acid is acidic and arginine is strongly basic. The clustering of these conserved amino acids suggests that they may be important in dsx protein specificity in sexual differentiation, relative to other DM domain proteins.

Figure 2 also shows alignment of the dsx dimer domain in *Nasonia*, close relatives and other insects, compared to the consensus pfam sequence for this region. As can be seen, there is considerable variation in the terminus of this domain, in both length and sequence. Because the dsx dimer is an alpha-helical motif (Bayrer *et al*., 2005), structure conservation rather than amino acid conservation is expected. Nevertheless, *Nasonia* and relatives retain a nine amino acid sequence in the middle of the dsx dimer region (MLYVILKDA) that is also mostly conserved in Diptera and *Tribolium*, but not in *Apis* or *Bombyx*. *D. melanogaster* shows the greatest similarity to the pfam sequence, but this is likely to reflect the contribution of *Drosophila* and other Diptera to determination of this consensus.

We have also compared the sequence of NV and its sister species, NG, *Nasonia longicornis* (NL), and *Nasonia oneida* (NO), as well as two closely related wasps, *Trichomalopsis sarcophagae* and *Trichomalopsis dubius*. The species dsx differ in length of the C terminus for the predicted female-specific protein. A 16-amino-acid long extension in the female-specific protein appears to be the ancestral condition, as it is shared amongst two *Nasonia* species, NV and NL, and *Trichomalopsis* (Fig. 2). In NG, the female transcript contains a stop codon that truncates the protein 12 amino acids relative to the ancestral form. A single deletion in NO creates a frameshift in this tail, resulting in a 32-amino-acid long extension in the females that has a completely different amino acid composition (Fig. 2). These results indicated accelerated evolution in the female dsx in these wasps and low purifying constraints. It is in agreement with recent work by Yang *et al*. (2008) showing that the C-terminal 'tail' of the dsx protein in *D. melanogaster* does not play an important role in dimer formation or intersex binding of the female protein. Intersex and the female form of dsx function together to repress male sexual differentiation in *Drosophila* (Baker & Ridge, 1980).

Discussion

We have described the *Nasonia dsx*, an orthologue of the *Drosophila* transcription factor gene *doublesex*. Evidence that *Nasonia dsx* is the *Drosophila dsx* orthologue is: (1) *Nasonia dsx* has a amino-terminus DM domain that clusters phylogenetically with highly conserved dsx DM domains from other insect species, as well as an oligomerization domain (dsx dimer); (2) *Nasonia dsx* is expressed as male and female specific spliceforms; (3) expression of *Nasonia dsx* in phenotypically variable gynandromorphic mutants matches their sexual phenotype differentiation, consistent with a role of *Nasonia dsx* in sex determination. Research on the sex-determining mechanism of *D. melanogaster* has shown that the transcription factor-coding gene *dsx* is the major switch at the base of the sex determination cascade, which subsequently determines somatic sex determination (Baker & Wolfner, 1988; Raymond *et al*., 1998). Here we have confirmed that in *Nasonia* the *dsx* gene is conserved, both in functional domains and in sex-specific splicing, indicating a similar major switch role in *Nasonia* sex determination.

In *D. melanogaster*, males can be considered the default gender, as the male-specific variant of *dsx* splicing requires no active upstream gene activity. This male default situation also seems to apply to the *Nasonia* sex-determining system, because the female specific variant of *dsx* requires an additional splicing event. However, in *Bombyx*, the female splice variant is the default because of suppression of the male splice variant (Suzuki *et al*., 2001). Therefore, without knowledge of upstream regulatory factors, the default gender in *Nasonia* remains unknown.

In *Drosophila*, the active specification of the sex-determining cascade ultimately leading to sex-specific splicing of *dsx* has long been considered the ratio of X chromosomes to autosomes (X : A). However, recent evidence indicates that the X-chromosome dosage rather than the X : A ratio is the key factor in sex determination in *Drosophila* (Erickson & Quintero, 2007). This dosage effect is exemplified through the timing of blastoderm formation: haploid embryos undergo an extra nuclear division cycle that prolongs the period in which X-encoded signal element proteins are expressed, whereas triploid embryos cellularize a cycle earlier than diploids. Erickson and Quintero (2007) recognize that sex determination in haplodiploids may work in a similar way. Crozier (1971) proposed that a chromosomal-cytoplasmic (maternal effect) balance could be the distinguishing signal between haploid and diploid embryos. Kamping *et al.* (2007) found evidence for a strong maternal effect on *Nasonia* sex determination. If haploid embryos develop slower than diploid embryos, a differential dosage effect of the chromosomal constitution of the embryo may tilt the balance towards males.

Sex-determining mechanisms vary greatly at the chromosomal level, ranging from male heterogamety (XX–XY) to female heterogamety (ZZ–ZW) to haplodiploidy without heteromorphic sex chromosomes. The underlying genes for sex determination are organized in cascades and can also vary amongst closely related species or even within species (Werren & Beukeboom, 1998). Nevertheless, there

is now ample evidence for evolutionary conservation of *dsx* at the base of these cascades in holometabolous insects. During evolution, genes appear to be added to the top of the cascade (Wilkins, 1995), but the responsible selective forces are not well understood.

We do not yet know which genes regulate alternative splicing of *dsx* in *Nasonia*, although *transformer* (*tra*), or a hymenopteran homologue, is a likely candidate. The *Drosophila* transformer and transformer-2 (tra2) proteins bind to regulatory elements, a 13-nucleotide sequence repeated six times, to activate the female-specific splicing of the *dsx* primary transcripts (Inoue *et al*., 1992). We have not been able to identify these regulatory elements in *Nasonia*, and they are not present in *Bombyx* (Ohbayashi *et al*., 2001) and *Apis* (Cho *et al*., 2007) either. Recently, the *feminizer* (*fem*) gene was characterized in the honeybee sex determination pathway. *fem* might have a function comparable to *tra*. It was shown that knockdown of the female form of *fem* results in diploid male bees (Hasselmann *et al*., 2008). Sex in honeybees is determined by complementation of two alleles of the *csd* gene (Beye *et al*., 2003). *fem* is the ancestral paralogue of *csd* and is also located near the *csd* locus (Hasselmann *et al*., 2008). Molecular details of how *fem* and *csd* regulate *dsx* are not yet known. Interestingly, a *fem* homologue is also present in the *Nasonia* genome (Hasselmann *et al*., 2008; E. C. Verhulst, unpubl. data). As sex determination in *Nasonia* does not depend on heterozygosity, a system with the *csd* gene as the primary regulator of *dsx* can be ruled out in this species. Nevertheless involvement of a *tra* homologue like *fem* seems probable. Currently we are analysing the involvement of the *Nasonia fem*/*tra* homologue in the sex-determining pathway in this genus.

Many models for sex determination in *Nasonia* have been proposed (reviewed in Cook, 1993 and Beukeboom, 1995). Sex determination in *Nasonia* is currently viewed as an interaction of maternal effects, chromosome dosage and genomic imprinting (Beukeboom *et al*., 2007b). Identification of the upstream genes to *dsx* in *Nasonia* is needed for a better understanding of its sex determination. This will also provide more insights in the evolution of sex determination in insects in general and that of Hymenoptera in particular.

Experimental procedures

Nasonia *rearing, strains and gynandromorphy induction*

Nasonia and the closely related genus *Trichomalopsis* were reared at 25 °C on *Sarcophaga bullata* or *Calliphora vicina* pupae under constant light. Strains of four *Nasonia* species, AsymCX or WM114 (*N. vitripennis*), RV2X(u) (*N. giraulti*), IV7X (*N. longicornis*), NONYBr36/11 (as yet undescribed *N. oneida*), and two *Trichomalopsis* species, *T. sarcophagae* and *T. dubius* were studied. In addition, the *N. vitripennis* strain HiCD12 was also investigated, which produces gynandromorphic individuals from unfertilized haploid eggs. Culturing details of the HiCD12 strain are described in Kamping *et al.* (2007) and Beukeboom *et al.* (2007a). In short, high temperature (31 °C) was used to induce maximum gynandromorph production as follows: virgin females were kept at 25 °C for 2–3 days with fly pupae for host feeding, hosts were removed and females were left at 31 °C for 12 h, after which they were given new hosts for oviposition during 12 h at 31 °C, and parasitized hosts were put at 25 °C for completion of *Nasonia* offspring development. Emerging gynandromorphs were selected based on their morphological resemblance to either males or females.

Tissue dissection and RNA purification

Male or female RNA was isolated from *Nasonia* tissue from a variety of life stages including embryos, larvae, pupae and adults, and body parts including head, thorax, abdomen, and wing and leg imaginal discs. Tissue was dissected under RNAse free conditions in 1 × phosphate-buffered saline. After dissection, tissue was placed immediately on dry ice and if necessary stored at –80 °C until RNA was isolated. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) or Invisorb Spin Tissue RNA Purification Kit (Invitek, Berlin, Germany). Poly(A) RNA was isolated using the Dynabeads mRNA DIRECT Kit (Dynal Biotech, Oslo, Norway) by the mini volumes protocol. When necessary, RNA was quantified using a Qubit fluorometer (Invitrogen) and a Quant-iT RNA Assay Kit (Invitrogen) or a ND-1000 Spectrophotometer (Nanodrop Technologies, Oxfordshire, UK). All steps were performed according to the respective manufacturer's protocols.

Exonic mRNA composition determination by RT-PCR and 5′and 3′RACE

Poly(A) RNA or total RNA were reverse transcribed using SuperScript III (Invitrogen) and Oligo(dT)12–18 primer (Invitrogen) or using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) and 3′ RACE adapter supplied with FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA). Both methods yielded similar results. The resulting cDNA was used for PCR with Taq polymerase (Invitrogen) under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; and 72 °C for 10 min. PCR screening for male vs. female spliceforms was carried out with forward primer dsdsx_FF (5′-CTG CCG AGT ATA CCA ATA CC-3′) and reverse primer dsdsx_FR2 (5′-GTA ATA GAA CAT TGG CAT CAA CC-3′). For full mRNA sequences, 5′- and 3′-RACE were conducted on 150 ng Poly(A) RNA from NV and NG using the GeneRacer Kit (Invitrogen). Reverse transcription was performed with a mixture of the included oligo-dT primer and a gene-specific primer dsx_1R (5′-TCG AGT CCT TTA AGA TTA CGT ACA T-3′).

Standard PCR and DNA sequencing

Genomic sequencing of all coding exons was performed for all four *Nasonia* species and the two *Trichomalopsis* species. DNA was obtained from single wasps (DNAeasy; Qiagen, Valencia, CA, USA) and long PCRs were performed with AmpliTaq (Invitrogen). Primers used are available from the authors upon request. Sequencing reactions were sent to the Cornell Biotechnology Resource Center or the University of Rochester Functional Genomics Center. Sequences were edited using the program Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA).

Sequence data from this article have been deposited with the GenBank Data Libraries under accession nos. FJ440989–FJ440998.

Bioinformatics and phylogenetic analyses

Five insect genomes, *D. melanogaster* (Build 5.1), *An. gambiae* (AgamP3), *T. castaneum* (Build 1.1), *Ap. mellifera* (Amel 4.0) and *N. vitripennis* (Nvit 1.0) were queried for genes containing DM domains using BLAST searches (Altschul *et al.*, 1990). The two conserved domains in the dsx protein, DM and dsx dimer, were aligned using the consensus sequences in the Pfam database as references (Finn *et al.*, 2006); pfam00751: DM DNA binding domain (DM domain) and pfam08828: doublesex dimerization domain (dsx dimer). Visual inspection of the alignment was performed with MACCLADE 4.08 (Maddison & Maddison, 2000).

Maximum parsimony (1000 tree-bisection-reconnection replicates) and neighbor-joining trees were inferred in PAUP 4.0 (Swofford, 2002) using amino acid sequences. As unambiguous alignment was restricted to the two conserved domains (DM domain and dsx dimer), only these regions were used in phylogenetic inferences. To construct a DM domain gene tree 67 aligned characters (42 parsimony-informative) were included, the entire DM domain plus the five flanking N-terminal amino acids and the 15 flanking C-terminal amino acids. To construct an insect phylogeny based on the *dsx* gene the same DM domain region was used combined with the dsx dimer region and the female-specific region. The region included the dsx dimer, the flanking five N-terminal amino acids and five flanking C-terminal amino acids, generating 83 additional characters. In total 138 characters were included in the dsx insect phylogeny (88 parsimony-informative). Branch support was accessed by 500 bootstrap replicates (Felsenstein, 1985).

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